# EVALUATION OF POTENTIAL BIOACCUMMULATIVE COMPOUNDS EXERTING ENDOCRINE-DISRUPTING ACTIVITIES IN WILD ANIMALS USING *IN VITRO* BIOASSAYS AND CHEMICAL FRACTIONATION

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**Introduction**

Environmental pollution by anthropogenic chemicals is one of the most pressing global problems, which have international attention as environmental concern and it is closely related with quality of life of human beings and wild animals. About fifty million chemicals have been produced industrially[1], and it is estimated that a huge number of artificial chemicals is released to the environment and will be accumulated in wild animals via food the web. If accumulated compounds in wild animals exert any toxicity such as endocrine-disruption, such bioaccumulative toxic chemicals should be determined and monitored. However, it is difficult for monitoring with only instrumental analysis to target on important compounds selected from the huge number of chemicals due to lack of toxicity information for all compounds. Therefore, our research has been focused on using *in vitro* bioassay together with chemical fractionation in an attempt to determine the existence and activity profile of potential bioaccumulative pollutants in wild animals for future monitoring studies.

In this study, accumulated compounds in blubbers and/or livers of Baikal seal, Common cormorant, Raccoon dog and Finless porpoise were extracted and subjected to chemical fractionation for subsequent *in vitro* bioassays. As *in vitro* bioassays, a panel of rat and human cell-based CALUX reporter gene bioassays was utilized to evaluate steroidal hormone-disrupting potency (androgen receptor (AR), estrogen receptor alpha (ERa), glucocorticoid receptor (GR), and progesterone receptor (PR)-mediated activities), dioxin-like toxicity (aryl hydrocarbon receptor (AhR) -mediated activity) and lipid metabolism-disrupting potency (peroxisome proliferator-activated receptor gamma (PPARg) -mediated activity) in fractionated extracts.

**Materials and Method**

*Samples.* Four higher-order wild animal species, Baikal seal (*Phoca sibirica*), Common cormorant (*Phalacrocorax carbo*), Raccoon dog (*Nyctereutes procyonoides*), and Finless porpoise (*Neophocaena phocaenoides*) were investigated in this study. Baikal seals were collected from Lake Baikal in Russia in 1992[2] and 2005[3], and their blubbers (1992: *n*=10, 2005: *n*=10) and livers (1992: *n*=6, 2005: *n*=10) were used in this study. Common cormorants were sampled from Lake Biwa of Japan in 2002[4]., Raccoon dogs from Kanagawa of Japan in 2001[5], stranded Finless porpoises from Japanese coasts in Hyogo, Ehime, Oita, and Nagasaki between 2005 and 2007, and their livers (each *n*=10) were analyzed in this study. All the applied samples were stored at -25 °C in the Environmental Specimen Bank (*es*-BANK) at Ehime University[6] until analysis.

*Extraction and chemical fractionation.* Using 25 chemicals including brominated flame retardants (BFRs), polyaromatic hydrocarbons (PAHs) and hormonal agents, an extraction and chemical fractionation scheme was established according to

fractionation characteristics of applied pure chemicals (Fig. 1), and applied to above-mentioned wild animal samples. Briefly, 10 g of target sample was extracted with an acetone/*n*-hexane (1/1 = v/v) using a rapid solvent extractor (SE-100, Mitsubishi Chemical Analytech, Japan) respectively. The extract was solvent-exchanged into *n*-hexane as the crude extract. A portion of the crude *n*-hexane extract was subjected to sulfuric acid treatment and applied to a sulfuric acid silicagel column. After elution with *n*-hexane, the extract was evaporated and dissolved in DMSO as "Persistent fraction". On the other hand, using chemical fractionation methods such as DMSO/*n*-hexane dispensation, silicagel column treatment and the liquid-liquid extraction, the remaining portion of the crude *n*-hexane extract was fractionated into 4 fractions dissolved in DMSO: "Strong hydrophobic fraction", "Moderate hydrophobic fraction", "Mild hydrophobic fraction", and "Weak hydrophobic fraction" according to the hydrophobicity of compounds contained in the crude extract as shown in Fig. 1. A portion of each fraction was taken-up and mixed by type of fraction as pooled extract. All extracts were stored at 4 °C until CALUX assays.

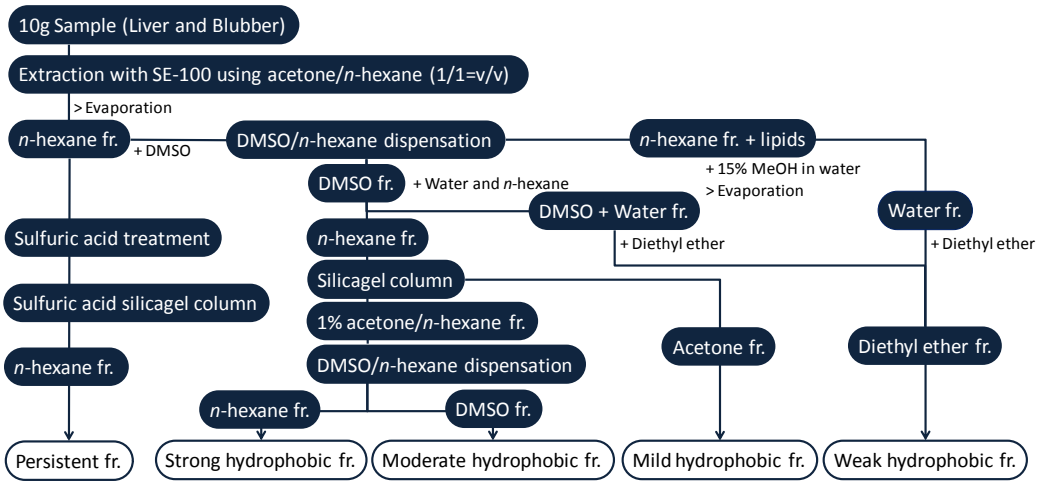


**Fig. 1 Extraction and chemical fractionation schemey**

*Screening of potential bioaccumulative compounds exerting endocrine-dirupting activities using CALUX assays.* Agonistic and antagonistic responses of all wild animal extracts were evaluated with human U2OS-*luc* cell line for AR-, ERa-, GR-, PR-, PPARg1-, and PPARg2-mediated responses and a DR-CALUX cell line for AhR-mediated response. The CALUX assay procedures have been described for U2OS-*luc*[7] and DR-CALUX[8].

Briefly, the cells were seeded into 96-well microplates with DF medium (without phenol red) supplemented with stripped (dextran-coated charcoal treated) fetal bovine serum (FBS) for U2OS-*luc* and alpha-minimal essential medium supplemented with FBS for DR-CALUX. After 24h of incubation at 37 °C under 5% $CO_2$, the medium was replaced by medium containing the extracts for agonistic response testing. Regarding antagonistic response experiment, the cells were exposed to not only the animal extracts but also a reference compound at $EC_{50}$ level in the same exposure medium. After 24 h of exposure, the medium was removed, and the cells were lysed in 30 µl of Triton-lysis buffer. After addition of luciferin solution, the luciferase activity was measured in a luminometer. On all plates, a dose-response curve or maximum concentration of the reference compound was also assayed for evaluation of the response. In this study, dihydrotestosterone (DHT), estradiol (E2), dexamethasone, Org2058, 2,3,7,8-TCDD, and rosiglitazone were used as reference agonists for AR-, ERa, GR, PR, AhR (DR)-, and PPARg-CALUX assay, whereas flutamide, tamoxifene and RU486 were used as reference antagonists on AR-, ERa-, and both GR- and PR-CALUX cells, respectively. All measurements were conducted in 3 wells. Experiments have been repeated 2 times independently at least.

**Results and discussion:**

In this study, accumulated compounds exerting endocrine-disrupting activities in blubber and/or liver of wild animals such as Baikal seal, Common cormorant, Raccoon dog, and Finless porpoise were evaluated with several CALUX assays. As a result, agonistic and/or antagonistic (synergistic) responses were observed in some extracts prepared from wild animals.

As an example, agonistic results on ERa-CALUX assay are shown in Fig. 2. The weak crude hydrophobic fractions derived from Common cormorant, Raccoon dog and Finless porpoise, but not Baikal seal had marked E2-like activity. Therefore, a dose–response result was depicted for these extracts as shown in Fig. 2. At least, obtained results indicate that some accumulated compounds in weak crude hydrophobic extracts from liver of Raccoon dog are full ERa agonist.

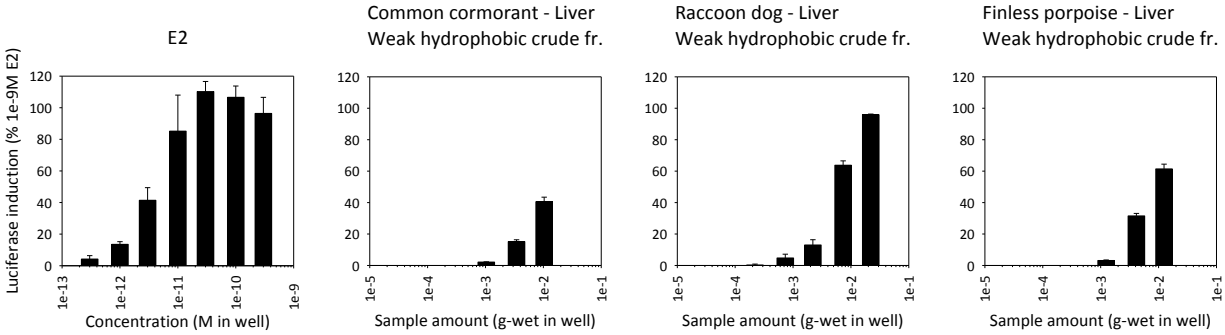


**Fig. 2 Results of ERa agonistic experiments for weak crude fractions from livers of Common cormorant, Raccoon dog and Finless porpoise. Values represent the mean ± S.D. from two independent assays.**

Regarding an example of antagonistic result in this study, antagonistic response observed in persistent fraction derived from blubber of Baikal seal was shown in Fig. 3. As depicted in Fig. 3-a, an antagonistic response was observed in a dose-dependent manner by co-exposure of persistent fractions from Baikal seal blubber and DHT (at $EC_{50}$ level) to the cells. In this study, potential antagonistic response at co-exposure with $EC_{50}$ level of DHT as reference compound was confirmed by co-exposure with 1000 x EC50 level of DHT. At co-exposure experiment with 1000 x EC50 level of DHT, no decrease of response in truly receptor-based antagonistic extract should occur. Furthermore, cytotoxicity by potential antagonistic extract was checked in the co-exposure experiment with EC50 level of DHT by using MTT assay. Results of co-exposure experiment with 1000 x EC50 level of DHT and MTT assay were respectively shown in Fig. 3-b and -c, indicating that persistent fraction derived from blubber of Baikal seal was a truly antagonistic extract.

Results of this study can be summarized as follows.With steroid receptors such as AR, ERa, GR and PR, truly antagonistic responses tended to be observed in some crude and persistent fractions. On the other hand, although ERa full agonistic responses were detected in weak crude hydrophobic fractions derived from Common cormorant, Raccoon dog and Finless porpoise, agonistic responses tended not to be detected in all kinds of extracts from wild animals. Potent AhR agonistic responses were shown in not only all persistent fractions and strong crude hydrophobic fractions but also some weak crude hydrophobic fractions. Antagonistic responses on DR-CALUX cells tended to be detected in most moderate and mild crude hydrophobic fractions. Regarding PPARg1- and PPARg2-CALUX, partial agonistic responses were observed for all crude fractions, but not persistent fractions.

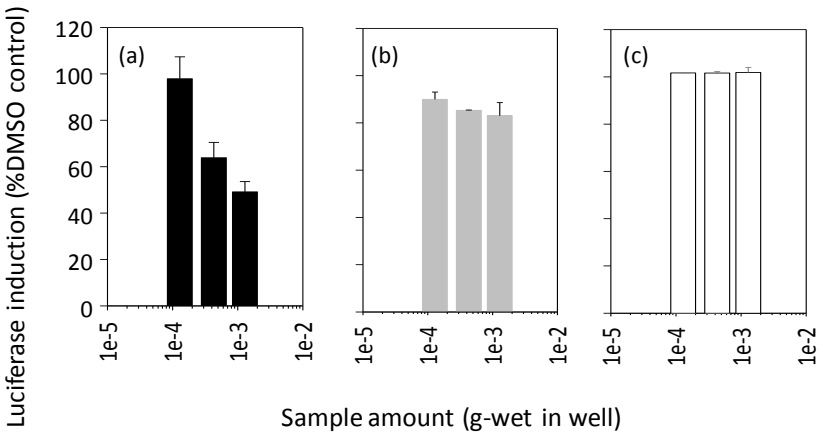


**Fig. 3 Results of AR antagonistic experiments for persistent fraction from blubber of Baikal seal collected at 2005. Co-exposure experiments extracts and DHT at $EC_{50}$ level (a) and 1000 x $EC_{50}$ level (b) to the cells, and MTT assay at $EC_{50}$ level of DHT (c). Values represent the mean ± S.D. from two independent assays.**

This study clearly indicates that potential bioaccumulative compounds exerting various endocrine-disrupting activities such as the steroidal hormone-disruption potency, dioxin-like toxicity and lipid metabolism-disruption potency exist in the investigated wild animals.

**Acknowledgements:**

This research was supported by Grants-in-Aid for Scientific Research (S) (No. 20221003) from Japan Society for the promotion of Science (JSPS) and Global Center of Excellence (GCOE) Program from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. The award of a JSPS Superlative Postdoctoral Fellowship for Researchers in Japan provided to Dr. Go Suzuki (20-5965) is acknowledged.

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